

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 March 2001 (29.03.2001)

PCT

(10) International Publication Number
WO 01/21827 A1

(51) International Patent Classification⁷: C12Q 1/00

(74) Agent: WOODBURN, Jill, L.; The Law Office of Jill L. Woodburn, L.L.C., 6633 Old Stonehouse Dr., Newburgh, IN 47630 (US).

(21) International Application Number: PCT/US00/25631

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date:
19 September 2000 (19.09.2000)

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

Published:

(26) Publication Language: English

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

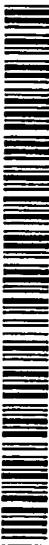
(30) Priority Data:
60/154,731 20 September 1999 (20.09.1999) US

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

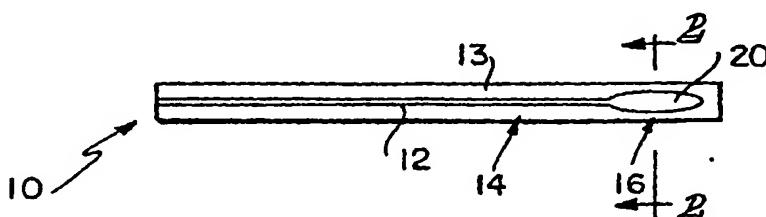
(71) Applicants (for all designated States except US):
ROCHE DIAGNOSTICS CORPORATION [US/US];
9115 Hague Road, P.O. Box 50457, Indianapolis, IN
46250-0457 (US). ROCHE DIAGNOSTICS GMBH
[DE/DE]; Sandhofer Strasse 116, 68298 Mannheim (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BUCK, Harvey, B.
[US/US]; 8147 Bay Brook Dr., Indianapolis, IN 46256
(US). ESSENPREIS, Matthias [DE/US]; 38775 Stoning-
ton Terrace, Fremont, CA 94536 (US).



(54) Title: SMALL VOLUME BIOSENSOR FOR CONTINUOUS ANALYTE MONITORING



WO 01/21827 A1

enzyme in contact with medium (24), and an electron transfer mediator. The fluid contacts sensors (10, 110, 210, 310, 410) and at initially predetermined intervals intermittently applies a potential to electrode (12) sufficient to oxidize the mediator and sensing current through electrode (12) as a function of the duration of the applied potential. The applied mediator oxidizing applied potential is maintained for a period of time sufficient to determine the rate of change of current with time through electrode (12). The current flow is correlated with the current flow for known concentrations of the analyte in medium (24).

(57) Abstract: Sensors (10, 110, 210, 310, 410) and a method for detecting an analyte are described. Sensors (10, 110, 210, 310, 410) each have a volume of a hydrophilic medium (24) that retains an amount of analyte proportionate to the concentration of analyte in a biological fluid, electrodes (12) and a redox

SMALL VOLUME BIOSENSOR FOR CONTINUOUS ANALYTE MONITORING

5 Field of the Invention

This invention relates to sensors for detecting the presence or quantity of an analyte in a biological fluid. More particularly, this invention is directed to a sensor adapted particularly for indwelling or implanted use. Analyte concentrations are measured electrochemically in a contained detection retention volume of an analyte-permeable medium optionally separated from the biological fluid with a semi-permeable membrane.

10 Background and Summary of the Invention

There has been a significant research and development effort directed to the development of electrochemical sensors capable of detecting the presence and/or quantity of biologically significant analytes. Many, if not most of such analyte sensing devices are in the form of a test strip comprising a test fluid containment space pretreated with an analyte-dependent detection composition and electrodes for contact with test fluid delivered into the test fluid containment space. Electrical conductors extend from the electrodes to an area on the test strip for connection to a hand held or table mounted preprogrammed sensor reading device. Typically a biological fluid is delivered to the sample fluid containment area or volume and the sensor reading device is programmed to apply a predetermined potential to the electrodes after a predetermined period of time following delivery of the fluid sample to the sample containment space. Current flow is then measured responsive to said applied potential to provide an indication of the presence and/or concentration of the target analyte.

25 Some of such electrochemical sensors are constructed to prevent direct contact of the sample fluid with the electrodes by covering the electrodes with a semipermeable membrane or gel matrix material, which is insoluble in the test medium and permeable at least to the analyte of interest when in contact with said test medium.

30 There is a continuing need for the development of commercially feasible, multi-/continuous-use sensors for biologically significant analytes. In particular, there is need for development of biological sensors capable of being implanted or injected into a patient for periods ranging from several hours up to several weeks, months, or years and designed to

-2-

provide accurate results without removal or recalibration to compensate for changes in diffusional properties of membrane components or for losses of enzyme activity and/or electron mediator elements. Such sensors would find application as components of artificial organs, for example an artificial pancreas, requiring continuous and/or regular monitoring of patient glucose 5 levels. Such devices could also find use as reusable sensors for measuring analyte concentrations in bodily fluids *in vitro*, such as the analytical situations encountered by commercial labs performing analysis on patient fluid samples.

There are unique problems presented by the design and construction of biological sensors capable of repeated use *in vitro* and/or continuous use *in vivo*. Indeed, inherent in such 10 functional requirements is the condition that the functional chemical component of the sensor be confined, *i.e.* not released from the sensor into the sample fluid during repeated and/or continuous use. The retention of the "active" chemical/electrochemical components of the biosensor can be accomplished by one of several techniques, alone or in combination. Thus the active components can be immobilized, for example, by covalent bonding to non-leachable 15 components of the biosensor or by confining the biologically/electrically active components in a testing zone or volume by means of a membrane permeable at least by the analyte, but not by the contained, optionally covalently bound, enzymes, coenzymes, and/or electron mediators.

The implantable and/or reusable biosensors in accordance with the present invention are designed to retain the active sensor-dependent chemical components, typically in a hydrophilic 20 matrix in an analyte retention volume. The active electrochemical species that cooperate in the sensor responsive to an applied potential to provide a current flow signal proportionate to the concentration of analyte diffused into the retention volume can optionally be covalently bound to non-leachable components of the retention volume including, but not limited to, an electrode of an electrode system, a wall of the enclosure portion of the sensor for defining, at least in part, the 25 retention volume, to microspheres or other microparticulate solids contained in the retention volume, to the retention volume contacting the side of a membrane, or to polymer components of the retention volume matrix. Alternatively the enzyme(s), the enzyme cofactor(s) and the electron mediator(s) can be selected to have a molecular weight sufficiently high to preclude any substantial diffusion of such components from the retention volume into the biological fluid 30 being analyzed.

-3-

In one aspect of this invention the retention volume medium, alternatively denominated the "depletion volume medium" is in contact with the electrode system comprising an electrode capable of receiving electrons from or delivering electrons to the enzyme(s) via the electron mediator(s). Conductor elements extend from the electrode to a point on the device for allowing 5 electrical communication of the electrode with a programmable controller. The controller can be programmed to apply a predetermined potential sequence to the electrode system including variable potential including either a mediator oxidizing potential or mediator reducing potential, variable pulse width and variable pulse intervals. The controller is also capable of sensing current flow responsive to applied potential(s) to the electrode system and comparing such data 10 with control data previously obtained for said system to calculate and report analyte concentrations in the biological sample being analyzed and, optionally, to use such data to sense the performance status of the device and use such for modifying the then existing potential sequence protocol to optimize device function. Thus, for example, the sensor control can be modified periodically to adjust for differences in analyte diffusion efficiency across the 15 membrane and/or changes in concentration of the active electron mediator and/or enzyme component(s) of the device without use of classical recalibration techniques.

In one embodiment of the invention the retention volume is defined or enclosed, at least in part, by an analyte-permeable membrane and the ratio of the retention volume to the surface area of the semipermeable membrane defining that volume, at least in part, is less than 2mm, 20 more preferably less than 1mm. The low volume to surface area ratios are preferred in that they improve the rate of diffusional equilibrium between the fluid being tested and the retention volume medium, and thereby it works to minimize the refractory period (the recovery period) of the sensor.

In one preferred embodiment the enzyme component is selected so that it is substantially 25 not capable of transferring electrons to or from any endogenous substance other than said analyte. Under such conditions the enzyme reaction responsible for providing a signal of analyte concentration cannot take place without a predetermined threshold potential being applied to the electrode system. The sensor can therefore be turned off to stop enzyme activity, optionally following a pulse of reducing potential to "deactivate" the mediator, and allow predictable

-4-

concentration-gradient-based diffusion to work to rapidly "reset" the analyte concentration in the analyte detection/retention volume for the next programmed pulsed potential detection sequence.

In another embodiment of the present invention there is provided a method for monitoring analyte concentration using the sensor of this invention by contacting the sensor with the biological fluid being analyzed. Initially at predetermined intervals a potential is applied intermittently to the electrode system sufficient to oxidize the electron mediator in the retention volume, and the current flow through the electrode is sensed as a function of the duration of the applied potential. The applied mediator oxidizing potential is maintained at least for a period of time sufficient to determine the rate of change of current through the electrode as a function of duration of the applied potential. Values for the sensed current are correlated with values of current flow for known concentrations of the analyte. Alternatively the sensing protocol can comprise adjusting the potential to establish a predetermined current flow and thereafter sensing the rate of change of potential required to maintain said current flow for a predetermined time period.

In another embodiment the analyte concentration in a biological sample is measured as a function of the time dependent concentration of analyte in the retention volume following analyte depleting potential pulses. The rate of recovery concentration in the retention/depletion volume can be readily correlated with analyte concentration in the biological fluid contacting the sensor. The "diffusion status" of the membrane can be checked by a preprogrammed sequence from time-to-time during sensor use and numerical values associated with the sensed status can be used as input to modify the preprogrammed pulse sequence algorithms for subsequent sensor operation.

These and other features of the invention are described hereinbelow with reference to the drawings and the best mode known for carrying out the invention.

25

Brief Description of the Drawings

Fig. 1 is a plan view of a sensor in accordance with the present invention.

Fig. 2 is a cross-sectional view of the sensor along lines 2-2 of Fig. 1.

Fig. 3 illustrates a cross-sectional view similar to Fig. 2 but the electrode is of different construction.

-5-

Fig. 4 is similar to Fig. 2 illustrating a cross-sectional view of a sensor in accordance with this invention having a diffusion-limiting membrane with large pores.

Fig. 5 is similar to Fig. 4 illustrating a cross-sectional view of a sensor embodiment of this invention wherein diffusion into the retention volume is via pores on the periphery of an otherwise non-permeable membrane component.

Fig. 6 is a graphic representation of a pulse sequence in which two pulses of oxidative potential of different duration are applied to the sensor, interspersed with recovery intervals with reducing potential.

Fig. 7 is a graphic representation of a pulse sequence in which the duration of the intervals between the pulses is changed

Figs. 8-9 are graphic representations of measurement protocols, which combine changes in the pulse interval with changes in the pulse width.

Fig. 10 is similar to Fig. 1 and is a plan view of a sensor in accordance with this invention.

Fig. 11 is a graph showing the measured response for the Pyrrole-3-acetic acid/Mediator/glucose dehydrogenase (GDH) sensor.

Fig. 12 is a graph showing the measured response for the Pyrrole-3-carboxylic acid/mediator/GDH sensor.

Fig. 13 is a graph showing the response of the Pyrrole-3-acetic acid/Mediator/GDH and the Pyrrole-3-carboxylic acid/mediator/GDH sensors.

Detailed Description of the Invention

The electrochemical sensor of this invention is designed to provide signals indicative of analyte concentration in a biological fluid. The sensor comprises an electrode in contact with a low volume of a hydrophilic medium for retaining an amount of analyte proportionate to the concentration of analyte in a biological fluid in contact with the sensor. The medium is selected to allow facile analyte diffusion through said medium alone or upon hydration of said medium prior to or consequent to sensor use. The sensor comprises an enclosure for the volume of hydrophilic medium. The enclosure is formed to expose the hydrophilic medium to the biological fluid so that analyte in the biological fluid diffuses into the hydrophilic medium until

-6-

the concentration of analyte in the medium is equivalent to the concentration of analyte in the biological fluid. The rate of analyte mass diffusion into the retention volume is dependent on the analyte concentration gradient. The concentration of analyte in the retention/depletion volume may be measured electrochemically by cooperation of an electron mediator and a redox enzyme specific for the analyte, each forming part of or being in contact with the hydrophilic medium.

The hydrophilic medium can have a water concentration level less than, equal to or greater than the water content of the biological fluid. Thus the components of the hydrophilic medium including the enzyme and electron mediator components and a hydrophilic polymer can be used in construction of the sensor in a substantially dehydrated state, ready for rehydration prior to use or upon contact of the sensor with a biological fluid. It is important for sensor function that the analyte of interest is readily diffusible through the hydrophilic medium to enable a substantially homogeneous concentration of the analyte in the analyte retention volume, and a concentration that closely corresponds to analyte concentration in the biological fluid in contact with the sensor.

The sensor is constructed to have an enclosure or compartment for holding the analyte retention volume of the hydrophilic medium. The enclosure compartment is formed to expose the hydrophilic medium to the biological fluid when the sensor is in use. In one embodiment, the enclosure compartment is defined at least in part by a wall comprising an area of an analyte permeable membrane having a first side in contact with the hydrophilic medium and an opposite side for contact with the biological fluid when the sensor is in use. The analyte of interest, water, and other membrane permeable components of the biological fluid diffuses through the membrane and into the retention volume of hydrophilic medium until the concentration of analyte in the medium is proportionate to the concentration of analyte in the biological fluid contacting the analyte permeable membrane component of the sensor. It is appreciated that while a sensor in accordance with the present invention may include an analyte permeable membrane, such a membrane is not necessary to the operation of the sensor and as such is not required.

The sensor is constructed to provide an electrode in electrical contact with the hydrophilic medium. The electrode is typically formed of a conductive element such as carbon, silver, gold, platinum, palladium and the like and typically extends into or forms part of the walls of the

-7-

container or chamber for the analyte retention volume of hydrophilic medium. In one embodiment the electrode is formed of platinum and the retention volume is defined as the space overlying the electrode. In another embodiment, the electrode can be formed of a graphite powder and the retention volume is defined by the intraparticulate spaces and, in at least one embodiment, an overlying analyte permeable membrane. In another embodiment the electrode is a component of an electrode system comprising a reference electrode and optionally an auxiliary electrode, which may be different or identical to the reference electrode. The electrode system can also comprise conductor elements for providing electrical communication between the electrode components of the system and a programmable controller to control the electrical potentials in said electrode system and to sense current flow through at least one of said electrodes responsive to said electrical potentials. Typically the programmable controller is constructed as a separate unit with electrical connectors adapted particularly for electrical communication between the controller and the electrode system of the sensor. The controller is typically a hand-held or table mounted unit capable of being reversibly connected to one or more biosensors and having data storage and data display elements.

The sensor for electrochemical analysis in accordance with this invention further comprises a redox enzyme and an electron mediator in contact with the hydrophilic medium. The enzyme is selected for its capacity to oxidize or reduce the analyte of interest. The enzyme is preferably selected as well for its lack of capacity for transferring electrons to or from substances other than said analyte that are capable of diffusing from the biological fluid into the analyte retention volume. Non-limiting examples of suitable enzymes include pyrroloquinoline quinone (PQQ) -dependent glucose dehydrogenase (GDH) (EC 1.1.99.17) or Hydroxybutyrate dehydrogenase (HBDH) (EC 1.1.1.30). Dehydrogenase enzymes for other diffusible analytes are known in the art and can be substituted dependent on the analyte of interest.

The electron mediator can be selected from any of a wide variety of electron mediators capable of facilitating transfer of electrons between the redox enzyme and a sensor electrode in contact with the medium containing or in contact with said enzyme and said mediator. A non-limiting example of a suitable mediator includes osmium (bis-bipyridyl) pyridinium chloride. It is appreciated, however, that a number of commercially available mediators, non-limiting examples of which are described in U.S. Patent No. 5,589,326, the disclosure of which is

-8-

expressly incorporated herein by reference, may be used in accordance with the present invention. The enzyme and electron mediator can be entrapped in a polymer matrix on the electrode. Optionally, the enzyme and electron mediator can be selected to minimize their diffusion through an analyte permeable membrane during sensor use or can be covalently bound 5 to the walls of the enclosure or to hydrophilic polymer components of the retention medium to minimize, if not prevent, their diffusion from the retention medium volume through the membrane and into the biological fluid during sensor use.

Any of a wide variety of hydrophilic polymers, typically having a molecular weight in excess of 5000 Daltons and having polyanionic, polycationic, or polyhydric functionality can be 10 used as a carrier or carrier matrix forming part of the hydrophilic medium component of the sensor. Examples of such polymers include cellulosic polymers such as cellulose acetate, hydroxy ethyl cellulose, polyethylene glycols, synthetic or natural gums such as guar or xanthan gums, alginic acid, poly (meth) acrylic acids and copolymers of acrylic acids and acrylic esters, glycosaminoglycans, and the like which polymers can be used. Additionally, electrically 15 polymerized matrices from monomers such as pyrrole-3-acetic acid and pyrrole-3-carboxylic acid can serve as the hydrophilic matrix and/or enzyme entrapping matrix.

Such hydrophilic polymers can be used alone or in combination to provide a hydrated or hydratable matrix through which the targeted analyte is readily diffusible. Further, such polyfunctional hydrophilic polymers can be used to "anchor" or otherwise impair the diffusion of 20 the enzyme or electron mediator components of the medium to minimize loss of such components from the retention volume during sensor use.

Thus, art-recognized electron mediators having, for example hydroxy, carboxy or amino functionality for example ferrocene carboxylic acid, can be coupled using art-recognized ester-forming or amide-forming coupling methodologies to form the hydrophilic medium for use in 25 preparation of the present sensors. Additional non-limiting examples of mediator compounds include osmium-containing redox mediators such as those described in U.S. Patent No. 5,589,326, the disclosure of which is expressly incorporated herein by reference, and mediator compounds that can be tethered to a polymeric matrix, which include redox reversible imidazole-osmium complexes. Non-limiting examples of such complexes include osmium-bipyridyl 30 conjugates such as bis(bipyridyl) imidazolyl haloosmium complexes characterized by fast

-9-

mediation kinetics and low redox potential (+150 mV vs. Ag/AgCl). Another group of osmium containing mediators include tris(bipyridyl) osmium complex labeled electrochemically detectable conjugates. The redox enzyme and an electron mediator can thus be inherently non-diffusible or chemically coupled with the high molecular weight components of the medium to 5 render the enzyme and electron mediator components substantially not capable of diffusing through the analyte permeable membrane during sensor use in contact with a biological fluid.

The enzyme component of the sensor is typically of sufficient molecular weight that diffusional loss of that component through the semipermeable membrane is marginal over the typical period of sensor use. Such enzymes can be incorporated into the hydrophilic medium 10 during device construction, as, for example, an enzyme lyophilizate formed by freeze drying a solution of enzyme in the presence of a hydrophilic monomer, for example, maltose or trehalose, or other enzyme stabilizing hydrophilic composition. The lyophilized enzyme can be retained in said medium during sensor manufacture and storage in a dehydrated state until rehydration prior to or during initial use of said sensor, thereby providing longer sensor shelf life.

15 Electron mediator components of the sensors of the present invention are not critical except for the fact that they should be selected or modified, for example by covalent bonding of polymer components of the hydrophilic matrix or the hydrophilic medium, to prevent or minimize diffusional loss of the electron mediator component from the analyte retention volume medium during the course of sensor use. The prior art is replete with reference to a wide variety 20 of compounds including metal chelates and other metal complexes such as ferrocene, and more particularly carboxy ferrocene that can be readily coupled covalently to non-diffusible components of the hydrophilic medium.

The membrane components of the sensor constructs of this invention can be any 25 biocompatible analyte permeable membrane, including for example cellulose acetate, polyurethane, and polycarbonate. Other polymeric biocompatible membranes suitable for use in biosensor construction are also well known in the art and any of such art-recognized analyte permeable membrane/membrane materials may be used in manufacture of the present sensors. Example of analyte permeable membranes, and as well electron mediators and redox enzymes 30 are describe in U.S. Patent No. 5,264,105, the specification of which is expressly incorporated herein by reference.

-10-

In one embodiment of the invention the hydrophilic matrix is bound on the surface of at least one of the electrodes of the electrode system. The hydrophilic matrix comprises an electron mediator covalently bound to a non-diffusible or poorly diffusible hydrophilic polymer component of the matrix. The redox enzyme is bound to a polymer component of the hydrophilic matrix as well. A sensor of the present invention, preferably already includes the electron mediator and redox enzyme components, which are exposed to analyte present in the retention volume of the sensor. Initially at predetermined intervals a potential is applied intermittently to the electrode system sufficient to oxidize the electron mediator and the current flow through the electrode is sensed as a function of the duration of the applied potential. The applied mediator oxidizing potential is maintained at least for a period of time sufficient to determine the rate of change of current through the electrode as a function of duration of the applied potential. Values for the sensed current are correlated with values of current flow for known concentrations of analyte.

In another embodiment of the invention the hydrophilic medium comprises either a polymeric electron mediator or an electron mediator covalently bound to a non-diffusible or poorly diffusible hydrophilic polymer component of the medium. The redox enzyme is included as a stabilized lyophilizate or is covalently bound itself to a polymer component of the hydrophilic medium. The hydrophilic medium also comprises polyfunctional components that can be reacted with difunctional crosslinking agents contacted with the surface of said hydrophilic medium to form *in situ* an analyte permeable membrane on the surface of the hydrophilic medium. Thus for example a polyhydric polymer or a di- or trihydric, preferably high molecular weight, monomer component of the hydrophilic medium can be reacted, for example, with a polyisocyanate, for example a diisocyanate in the vapor phase, to form a polymer skin or membrane on the surface of the hydrophilic medium. The permeability of the membrane can be controlled by the length of exposure of the polyhydric medium surface to the multifunctional crosslinker. Thus, for example, 1,4-benzene diisocyanate can be vaporized in a chamber. Sensor constructs comprising the hydrophilic medium, preferably already including the electron mediator and redox enzyme components, having an exposed surface is introduced into the chamber for a period of time sufficient to form a biocompatible membrane on the surface of the hydrophilic medium to define in conjunction with other sensor components for example, a

-11-

simple planar non-conductive substrate, the enclosure for the analyte retention volume component of the sensor.

With reference to Fig. 1, there is provided in one embodiment of this invention a sensor 10 utilizing a thin-film gold electrode 12 on an inert substrate 14. In cross section (see Fig. 2) a sensing portion 16 includes gold electrode 12 on a surface 13 of inert substrate 14, spacer layers 18 and a diffusion-limiting membrane 20 having small or no pores. Membrane 20 can be formed as a separate sheet and applied to spacer layers 18 resting on gold electrode 12 and inert substrate 14 to complete definition of an enclosure for a depletion volume 22 filled with a hydrophilic medium 24. The enzyme and mediator may be immobilized, for example by entrapment in a hydrophilic matrix on the gold electrode 12. Additionally, it is contemplated that the enzyme and mediator may be immobilized by covalent bonding to a wall 26 of enclosure 22 or to overlying membrane 20 or such components can be freely diffusing and selected to have minimal membrane permeability. Diffusion-limiting membrane 20 can be selected from art-recognized analyte permeable membranes and adhered to a surface 28 of spacer layer 18 to complete enclosure 22 for the analyte retention/depletion volume. Alternatively the analyte permeable membrane can be formed in situ by cross-linking polyfunctional hydrophilic polymer and/or monomer components of the enzyme/mediator reagent layer. Still further, sensor 10 may be formed without membrane 20. It is understood that as used throughout the disclosure, that like reference numerals are used to denote like components.

With reference to Fig. 3, sensor 110 is provided in accordance with this invention. Sensor 110 includes an electrode formed of a porous/particulate carbon layer wherein the depletion volume is defined by the interstitial spaces between carbon/graphite particles (not shown). Thus a carbon-enzyme-mediator reagent layer 122 can be formed, for example by screen printing, a carbon/graphite suspension comprising a redox enzyme, and a nondiffusible or poorly diffusible, for example a polymeric, electron mediator in combination with one or more optionally non-electron mediating, polyfunctional polymers. The suspension is typically deposited on a conductor element (not shown) on an inert substrate. The diffusion-limiting analyte permeable membrane 20 can be applied, similar to that mentioned above, as a preformed polymer sheet applied and sealed over a surface 123 of the carbon electrode or membrane 20 can be formed by coating the exposed printed electrode having an intraparticulate polyfunctional

-12-

polymer matrix with a polymer in solution. See, for example, International Patent Application No. WO 98/17995 for non-limiting examples of polymer membranes for coating biosensors.

With reference to Fig. 4, which is similar to Fig. 1, sensor 210 of the present invention includes a diffusion-limiting membrane 220 that is formed to have large pores 223 for enhanced glucose diffusion. The mediator and enzyme components are preferably immobilized by entrapment in a hydrophilic matrix on electrode 12, by covalent bonding to nondiffusible polymeric components of hydrophilic medium 24, or to surfaces 26 of sensor enclosure 22 for the retention volume.

With reference to Fig. 5, there is provided another embodiment in the invention. Sensor 310 is similar to that illustrated in Figs. 1 and 4 with the exception that membrane 320 overlying hydrophilic medium 24 is nonpermeable itself. Membrane 320 is provided with peripheral porosity allowing diffusion of glucose or other analytes into the retention/depletion volume.

The biochemical sensor of this invention can be constructed in any form adapted for the intended use. Thus, sensors intended for repeated laboratory use can be constructed in the form of an elongated probe having the sensor element itself located at one end and electrical conductors connecting the electrode component of the sensing element to points of electrical attachment of the probe sensor to a programmable sensor controller. Alternatively the present electrochemical sensor can be constructed using art recognized micro scale manufacturing techniques to produce needle-like sensors capable of being implanted or injected into a site for indwelling sensor applications.

The electrochemical sensor of this invention can be utilized for monitoring analyte concentrations in biological fluids. The method comprises the steps of contacting the biological fluid with the sensor and at initially predetermined intervals intermittently applying a potential to the electrode sufficient to oxidize the electron mediator. The current passing through the electrode is then sensed as a function of the duration of the applied potential. The applied mediator-oxidizing potential is maintained for at least a period of time sufficient to determine the rate of change of current with time through said electrode. The sensed current flow is then correlated with current flow for known concentrations of said analyte in the retention/detection medium. Alternatively the sensor can be constructed to comprise at least a working electrode and a reference electrode and optionally an auxiliary electrode, which may be identical with the

-13-

referenced electrode. At initially predetermined intervals a potential is applied sufficient to establish a predetermined level of current flow between the working electrode and the auxiliary electrode. And the potential difference between the working and reference electrodes necessary to establish said level of current flow is measured and maintained for a period of time sufficient
5 to determine the rate of change of potential necessary to maintain said current flow through said electrode. The potential measurements are then correlated with potential measurements recorded for known concentrations of analyte in the biological fluid.

The applied potentials, the duration of the potential pulses, and the intervals between potential pulses are entered into a programmable controller used in conjunction with the sensor
10 for analyte measurements. In one embodiment of the invention the intervals between the intermittent applied potentials are less than the time necessary for the concentration of the analyte in the retention volume to equilibrate with that in the biological fluid in contact with the analyte permeable membrane. In another embodiment the intervals are increased incrementally for a series of applied potentials, and the concentration of the analyte and the biological fluid is
15 determined as a function of the rate of increase in analyte concentration in the retention volume. The intervals between applied potential pulses can be modified based on previous measurements to adjust for variations in sensor performance deriving from loss or degradation of redox enzyme and electron mediator components and/or change in diffusion characteristics of either the analyte permeable membrane or the retention volume medium. Alternatively the duration of the applied
20 potential pulse is modified based on previous measurements of sensed sensor performance status. In still another embodiment of sensor operation the intervals between measurements is substantially equal to or greater than the time required for the analyte concentration in the retention volume to equilibrate with that in the biological fluid.

Figs. 6-9 present graphic illustrations of sample measurement algorithms wherein the
25 potential of the electrode is controlled over a period of time to vary between a potential at which no oxidation of mediator occurs (E_0) to a mediator oxidizing potential (E_1) and a potential at which reduction of the mediator takes place (E_{-1}). The potential protocol to be applied in any given situation depends on sensor status, the form of the sensor, and the nature of the electron mediator and redox enzyme. The protocol for sensor operation can be optimized by empirical
30 measurement and observations by the skilled user.

-14-

Specifically, Fig. 6 shows a pulse sequence in which two pulses of oxidative potential of different duration are applied to the sensor, interspersed with recovery intervals with reducing potential. By comparing the current profile from the first pulse with that from the second, information on the rate of enzymatic turnover of the substrate, and the rate of electron diffusion within the sensor may be obtained. Between the oxidative potential applications, the reducing potential ensures that all of the mediator is returned to its initial state prior to the application of the next oxidizing potential.

Fig. 7 shows a sequence in which the duration of the intervals between the pulses is changed. By comparing the current observed from the second pulse to that from the first, information on the recovery time of the sensor can be gained. The recovery time yields information not only on analyte concentration, but also on diffusion into the hydrophilic matrix. Measuring the rest potential of the sensor between the potentiostatic pulses also provides information on the sensor recovery.

Figs. 8-9 demonstrate measurement protocols, which combine changes in the pulse interval with changes in the pulse width. The controller can select from a variety of sequences and durations of amperometric measurement intervals and recovery intervals to determine not only the analyte concentration, but also probe the condition of the sensor for enzyme activity, diffusion of substrate and mediator within the sensor, and diffusion of substrate into the sensor.

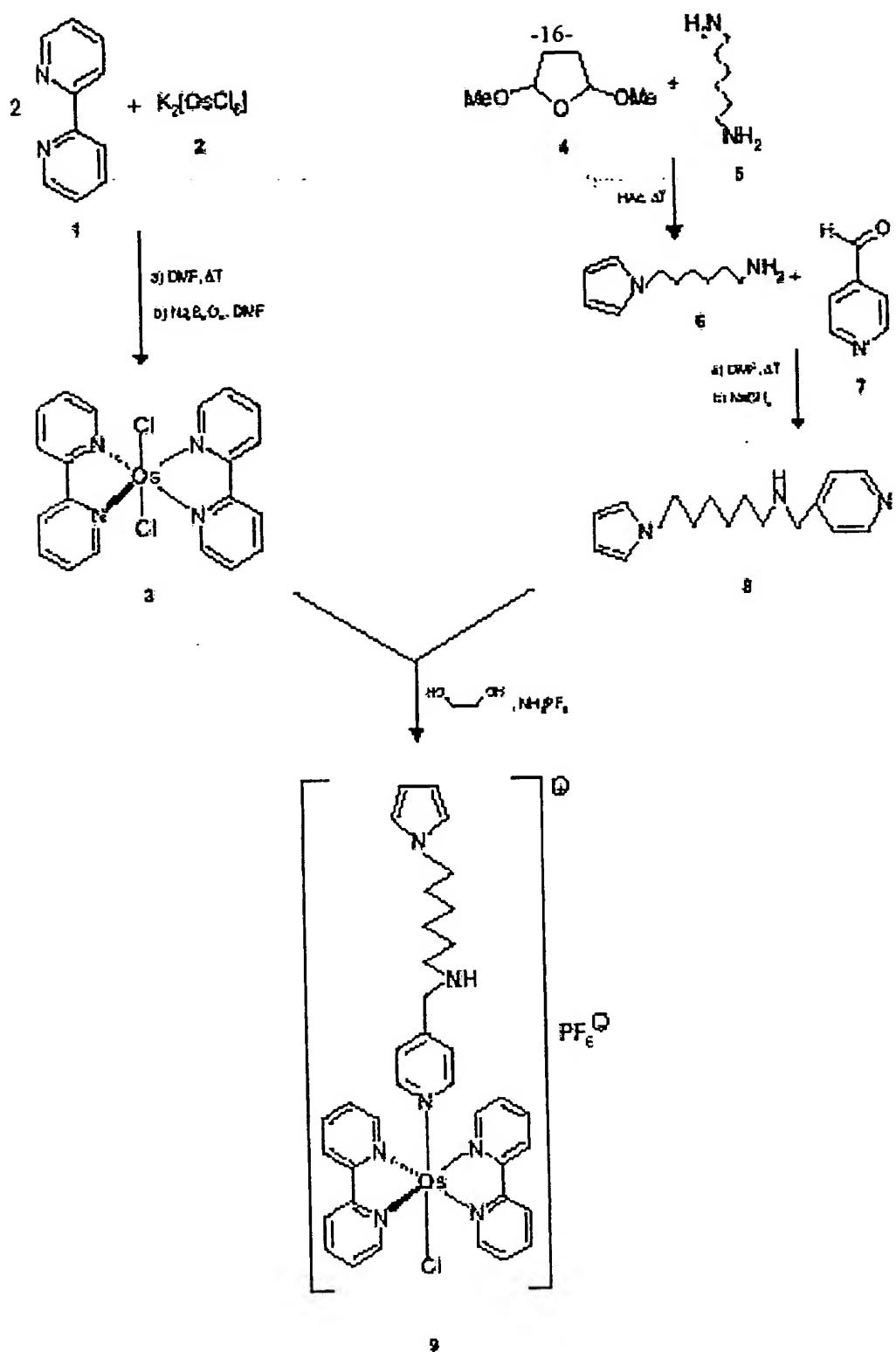
The nature and construction of the controller used with the present electrochemical sensor is not critical provided that the controller can be programmed to apply the appropriate potential pulse profiles to the sensor electrode or electrode system and to sense the current flow as a function of potential in time. A separate sensor status assessment protocol can be implemented in which the controller implements a protocol that allows computation of diffusion characteristics of the depletion volume and membrane from current data. The controller can be programmed to adjust measurement intervals and pulse widths based on computed diffusion times and determined substrate concentration. Both chronoamperometry and chronocolometry may be used to determine sensor status and substrate concentration. Ideally the controller should also be capable of applying a reducing potential to the electrode to reduce the mediator and thereby decrease consumption of analyte between measurements.

-15-

EXAMPLE 1:

A sensor in accordance with the present invention is formed similarly to sensor 10 of Figs. 1-2, except that it does not include membrane 20. The enzyme and electron mediator is entrapped in a polymer matrix on the electrode. This polymer matrix was prepared by 5 electropolymerization of pyrrole and pyrrole-mediator derivatives. This method entrapped the enzyme in a polymer matrix on the electrode, and, by incorporating mediator-derivatized pyrrole into the polymer, provided for an immobilized mediator for transfer of electrons within the sensor.

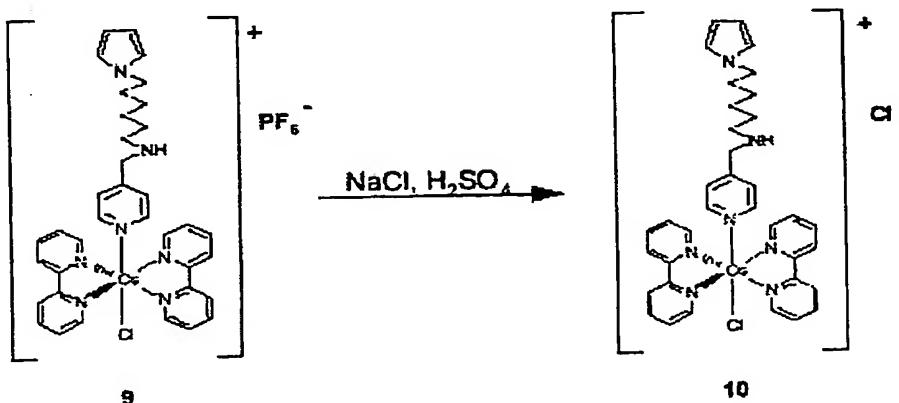
Platinum disc electrodes were suitable for preparing sensors in accordance with this 10 method. A mediator which was suitable for copolymerization in a matrix was prepared by the following reaction sequence:



Synthesis Of Pyrrole-Modified Osmium (Bisbipyridyl) Pyridinium Chloride

Pyrrole-modified Osmium (bisbipyridyl) pyridinium Chloride was prepared by the following reaction sequence:

5



Cleaning Procedure For Platinum Electrodes

The platinum disc electrodes were polished with Al_2O_3 -paste of decreasing roughness ($3\mu\text{m}$, $1\mu\text{m}$, $0.1\mu\text{m}$) on a polishing cloth and were washed with distilled water. Next, the electrodes were cleaned in an ultrasonic bath first in 10 M NaOH and then in 5 M H_2SO_4 - ten minutes each.

10

The electrodes underwent electrochemical cycling in oxygen-free 0.5 M H_2SO_4 :

1. Scan: -610 to + 1000 mV vs. Hg/HgSO_4 (100 mV/s, 10 μA)
2. Scan: -810 to + 1600 mV vs. Hg/HgSO_4 (100 mV/s, 10 μA)
3. Scan: -610 to + 1000 mV vs. Hg/HgSO_4 (100 mV/s, 10 μA)

15

The third scan was repeated until the cyclic voltogram showed a clean platinum surface and stayed constant.

The last step was the polarization at -210 mV for five minutes.

Platinization

Additionally, 4 ml of an oxygen-free 2 mM H_2PtCl_6 -solution was prepared under Argon 20 in a degassed electrochemical cell. Three voltammetric cycles from +500 to -400 mV vs. Ag/AgCl with a scan rate of 10 mV/s were applied. Then the electrode was washed under argon

-18-

with oxygen-free distilled water and stored under argon until use.

Polymerization Of Pyrrole/Mediator

5 A pyrrole film was polymerized to contain active Osmium (bis-bipyridyl) pyridinium Chloride as follows:

Co-polymerization was carried out with a mixture of 2mM pyrrole, 8mM (9) and 25 mM Tetramethyl ammonium perchlorate (as electrolyte) in 1:1 mixture of acetonitrile: water. 100 Pulses with potential / time of 0 V for 5 sec / 1.5 V 1 sec. were carried out. The polymerization was carried out in the absence of oxygen.

10 Polymerization Of Pyrrole-3-Acetic Acid/ GDH

In a solution of 50 mM Pyrrole-3-acetic acid, 0.05 mM PQQ, and 50 mM KCl in 0.1 M HEPES buffer, 5 mg/mL of GDH was dissolved. Following a 30 minute incubation to allow for reconstitution of the apoenzyme, the solution was polymerized with potential pulse profile consisting of 20 pulses of 0 V. for 5 sec / 1.2 V for 1 second.

15 Polymerization Of Pyrrole-3-Carboxylic Acid/ GDH

20 In a solution of 50 mM Pyrrole-3-carboxylic acid and 50 mM KCl in 0.1 M Hepes buffer, 5 mg/mL of sGDH was dissolved. The solution was polymerized with potential pulse profile consisting of 20 pulses of 0 V. for 5 sec / 1.2 V for 1 second, or alternatively of 0 V. for 5 sec / 1.4 V for 1 second, or also of 0 V. for 5 sec / 1.6 V for 1 second. Potential impacted the sensor performance considerably, indicating the dependence on polypyrrole film properties.

Polymerization Of Pyrrole-3-Acetic Acid/Mediator/ GDH

25 In a solution of 50 mM Pyrrole-3-acetic acid, 10 mM Osmium modified pyrrole derivative 10, 0.05 mM PQQ and 50 mM KCl in 0.1 M HEPES buffer 5 mg/mL of sGDH was dissolved. The solution was polymerized with potential pulse profile consisting of 20 pulses of 0 V. for 5 sec / 1.4 V for 1 second.

-19-

Polymerization Of Pyrrole-3-Carboxylic Acid/Mediator/ GDH

In a solution of 50 mM Pyrrole-3-acetic acid, 10 mM Osmium modified pyrrole derivative 10, 0.05 mM PQQ and 50 mM KCl in 0.1 M HEPES buffer 5 mg/mL of sGDH was dissolved. The solution was polymerized with potential pulse profile consisting of 20 pulses of 0.5 V. for 5 sec / 1.4 V for 1 second.

Enzyme electrodes were washed with deionized water. It is appreciated, however, that the enzyme electrodes may be washed with a buffer solution or, if necessary, with 3M KCl solution to remove adsorbed GDH and afterwards stored in 0.1 M phosphate buffer pH 7. If 10 electrodes are stored over-night they are kept at 4°C otherwise at RT.

Evaluation Of Sensor Response To Glucose

Sensor response was measured by placing the sensor into a stirred PBS (Phosphate buffered saline) buffer along with a reference electrode and a counter electrode. A potential (E) 15 sufficient to oxidize the immobilized mediator was applied, and the solution stirred for a time (t) until the current (I) stabilized to a low value. See Fig. 11. Aliquots of 1 M glucose solution in PBS were added in a step-wise manner, as shown by arrows in Fig. 12, to the PBS buffer to increase the concentration of glucose in the stirred PBS buffer solution, and the current (I) was measured on the plateau region of the response curve.

20 Referring now to Fig. 13, the response was measured for the Pyrrole-3-acetic acid/Mediator/GDH and pyrrole-3-carboxylic acid/mediator/GDH sensors. The response shows good electron transfer through the immobilized mediator in the polypyrrole film.

EXAMPLE 2:

25 A sensor 410 consists of conductors 12, 15 and reagents deposited on a flat polymeric substrate 14. Materials for encapsulation of the conductors 12, 15 are provided, and reagents that form a semipermeable biocompatible layer over the reagent-containing sensing area 16. See Fig. 10.

-20-

Processes and materials:

Substrate: Polyimid (such as Kapton® polyimide film, which is commercially available from E.I. DuPont de Nemours, Wilmington, Delaware, and Upilex® polimide film which is commercially available from UBE Industries Ltd, Japan) 0.005" (0.127 mm) thick with gold electrodes and conducting tracks.

5 Processing: Material is washed with water, acetone, and methylene chloride, then dried at 180° C for 20 hours. Material is placed in a vacuum chamber and metalized with 10 50 Å Chromium followed by 500 Å Gold. Metalized material is removed. A laminated photoresist is applied. The resist is exposed, and developed in an aqueous salt solution. Then the metal pattern is developed in a metal etchant 15 solution (typically HNO₃ / HCl). The patterned material is rinsed with water, and the remaining photomask is removed with a solvent (typically N-Methylpyrrolidone (NMP)). A photodefinable polyimid layer 2 um thick is applied with a spin coater, and solidified by baking at 80° C for 20 minutes. It is exposed and then developed with a solvent (NMP). It is then baked at 200° C for 15 30 minutes to harden the remaining polymer.

Reference/

20 Counter: A silver/silver chloride ink preparation consisting of suspension of silver particles, partially converted to silver chloride on their surfaces, in an organic solvent, i.e. cyclohexanone, with a polymeric suspending agent, such as alginate.

Processing: The ink mixture is applied to the opening in the polyimid and dried for 30 minutes 25 at 80° C to remove all solvent.

Sensor Area: The opening through the polyimid to the gold electrode is covered with a multilayer reagent area consisting of the following reagents.

-21-

Active

Enzyme Layer: A solution of Glucose Dehydrogenase/ pyrroloquinoline quinone (PQQ) (Enzyme Commission No. 1.1.99.17) and a redox polymer consisting of polyvinylimidazole with bis (bipyridyl) chloro-Osmium in phosphate buffer, 5 optionally with a polyhydric synthetic, natural or semisynthetic polymer, is applied to one electrode opening and dried. A solution of polyethylene glycol diglycidyl ether in aqueous buffer (i.e. 10 mM NaPO₄, 150 mM NaCl) is applied to the area, and allowed to dry at room temperature overnight. The electrodes are then rinsed in physiological saline solution and allowed to dry.

10

Biocompatibility

Layer: The entire structure is placed in a vacuum chamber. A plasma of diglyme is created in the chamber by introducing a low pressure vapor, and applying an RF field to dissociate the diglyme and cause polymerization. After 5 minutes, the 15 diglyme addition is stopped, and the plasma continued for a further 5 minutes. Then the vacuum is broken and the sheet removed from the chamber.

Post-

Processing: The sensors 410 are punched from the sheet and mounted into small polycarbonate holders which protect the sensing area and serve as the insertion 20 device. The packaged sensors are sealed into polyethylene bags. The bagged sensors are radiation-sterilized. Ten sterilized sensors are packed into a larger plastic box containing desiccant in the lid. The box is closed and packed into a cardboard outer pack that serves as the final consumer package.

25 This method for the fabrication of sensors suitable for use according to the current invention utilizes the so-called "wired-enzyme" technology for the immobilization of enzyme and mediator in active relationship within a sensor film. The GDH/PQQ substantially does not react with any endogenous substrate other than the analyte of interest. This allows the sensor to remain inactive, or "off" so long as the electrode is not regenerating the mediator to allow further 30 activity. Sensors produced by this method demonstrate very high sensitivity to glucose and very

-22-

high current density.

Although the invention has been described in detail with reference to a preferred embodiment, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.

What is claimed is:

1. A method for monitoring analyte concentration in a biological fluid by use of a sensor, the method comprising the steps of:
 - 5 providing a sensor comprising a volume of a hydrophilic medium for retaining an amount of analyte proportionate to the concentration of analyte in a biological fluid in contact with the sensor, an enclosure for said analyte retention volume, an electrode in contact with the hydrophilic medium, a redox enzyme in contact with the medium, and an electron transfer mediator for facilitating transfer of electrons between the enzyme and the electrode, the enzyme substantially not capable of transferring electrons to or from components of the biological fluid other than said analyte,
 - 10 contacting the biological fluid with the sensor and at initially predetermined intervals intermittently applying a potential to the electrode sufficient to oxidize the electron mediator and sensing current through said electrode as a function of the duration of the applied potential,
 - 15 maintaining the applied mediator oxidizing applied potential at least for a period of time sufficient to determine the rate of change of current with time through the electrode, and
 - 20 correlating the current flow with the current flow for known concentrations of said analyte in the retention medium.
2. The method of claim 1 wherein the intervals between the intermittent applied potentials are less than the time necessary for the concentration of the analyte in the retention volume to equilibrate with that in the biological fluids in contact with the enclosure.
3. The method of claim 2 wherein the analyte is glucose and the redox enzyme is a glucose dehydrogenase.
4. The method of claim 2 wherein the analyte is glucose and the redox enzyme is a PQQ-dependent glucose dehydrogenase.
5. The method of claim 2 wherein the electron mediator is osmium (bis-bipyridyl) pyridinium chloride.
6. The method of claim 2 wherein the redox enzyme and electron mediator are entrapped in a hydrophilic matrix on the electrode.
- 30 7. The method of claim 1 wherein the intervals are increased incrementally for a

-24-

series of applied potentials and the concentration of the analyte in the biological fluid is determined as a function of the rate of increase in analyte concentration in the retention volume.

8. The method of claim 7 wherein the intervals between applied potential pulses are modified based on previous measurement results.

5 9. The method of claim 7 wherein the duration of the applied potential pulse is modified based on previous measurement results.

10. The method of claim 1 wherein the interval is substantially equal to or greater than the time required for the analyte concentration in the retention volume to equilibrate with that in the biological fluid.

10 11. The method of claim 10 wherein the analyte is glucose and the redox enzyme is a glucose dehydrogenase.

12. The method of claim 10 wherein the analyte is glucose and the redox enzyme is a PQQ-dependent glucose dehydrogenase.

15 13. The method of claim 1 further comprising the step of applying a potential to the electrode sufficient to reduce the oxidized form of the electron mediator.

14. The method of claim 1 wherein the analyte is glucose and the redox enzyme is a glucose dehydrogenase.

15 15. The method of claim 1 wherein the analyte is glucose and the redox enzyme is a PQQ-dependent glucose dehydrogenase

20 16. The method of claim 1, wherein the sensor additionally comprises at least a reference electrode and an auxiliary electrode and further comprising the steps of intermittently establishing predetermined level of current to flow between said working electrode and said auxiliary electrode at initially predetermined intervals, and measuring the potential difference between said working electrode and the reference electrode; maintaining said level of current flow at least for a period of time sufficient to determine the rate of change of potential necessary to maintain said current through said electrode with time, and correlating said potential with the potential for known concentrations of said analyte in the biological fluid.

25 17. The method of claim 16 wherein the intervals between the intermittent potential measurements are less than the time necessary for the concentration of the analyte in the retention volume to equilibrate with that in the biological fluids in contact with the enclosure.

-25-

18. The method of claim 16 wherein the intervals are increased incrementally and the concentration of the analyte in the biological fluid is determined as a function of the rate of increase in analyte concentration in the retention volume.

19. The method of claim 18 wherein the intervals are modified based on previous 5 measurement results.

20. The method of claim 18 wherein the duration of current flow is modified based on previous measurement results.

21. The method of claim 16 wherein the interval is substantially equal to or greater than the time required for the analyte concentration in the retention volume to equilibrate with 10 that in the biological fluid.

22. The method of claim 1 further comprising a programmable controller in electrical contact with the electrode to control the electrical potentials at said electrode and sensing current through said electrode responsive thereto.

23. A sensor for use in electrochemical analysis of analyte levels in a biological fluid, 15 the sensor comprising:

a volume of a hydrophilic medium for retaining an amount of analyte proportionate to the concentration of analyte in a biological fluid in contact with the sensor,

an enclosure for said analyte retention volume,

an electrode in contact with the hydrophilic medium,

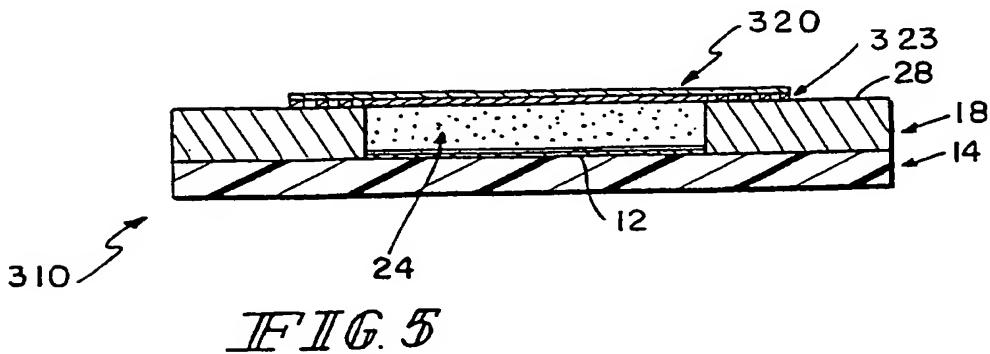
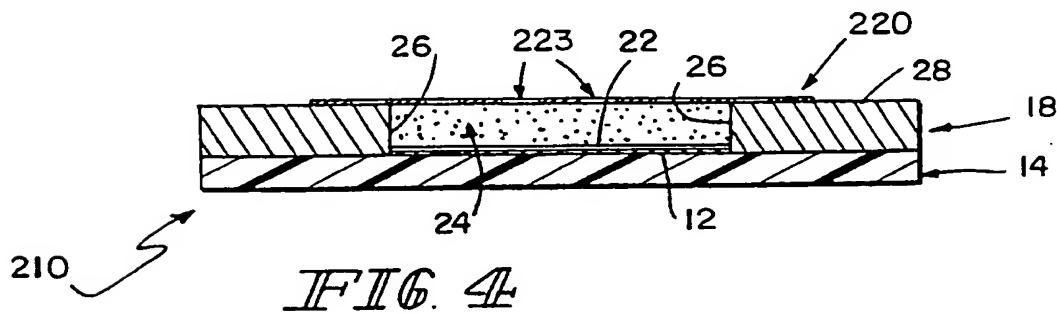
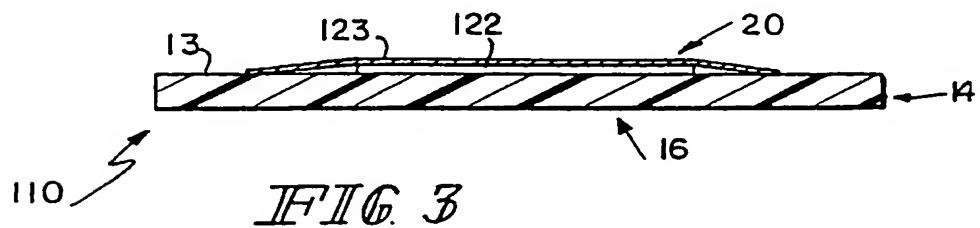
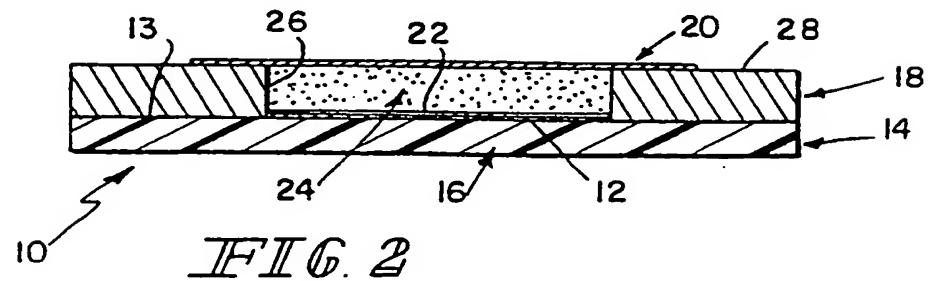
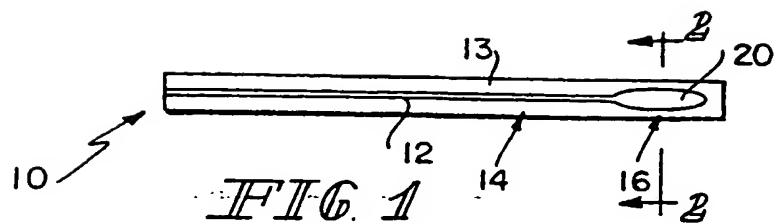
20 and a hydrophilic matrix on the electrode, the hydrophilic matrix comprising poly(pyrrole-3-acetic acid), an electron mediator, and a redox enzyme.

24. The sensor of claim 23 wherein the electron mediator is osmium (bis-bipyridyl) pyridinium chloride.

25. The sensor of claim 24 wherein the redox enzyme is a glucose dehydrogenase.

26. The sensor of claim 23 wherein the redox enzyme is a glucose dehydrogenase.

27. The sensor of claim 23 wherein the redox enzyme is a PQQ-dependent glucose dehydrogenase.



2/4

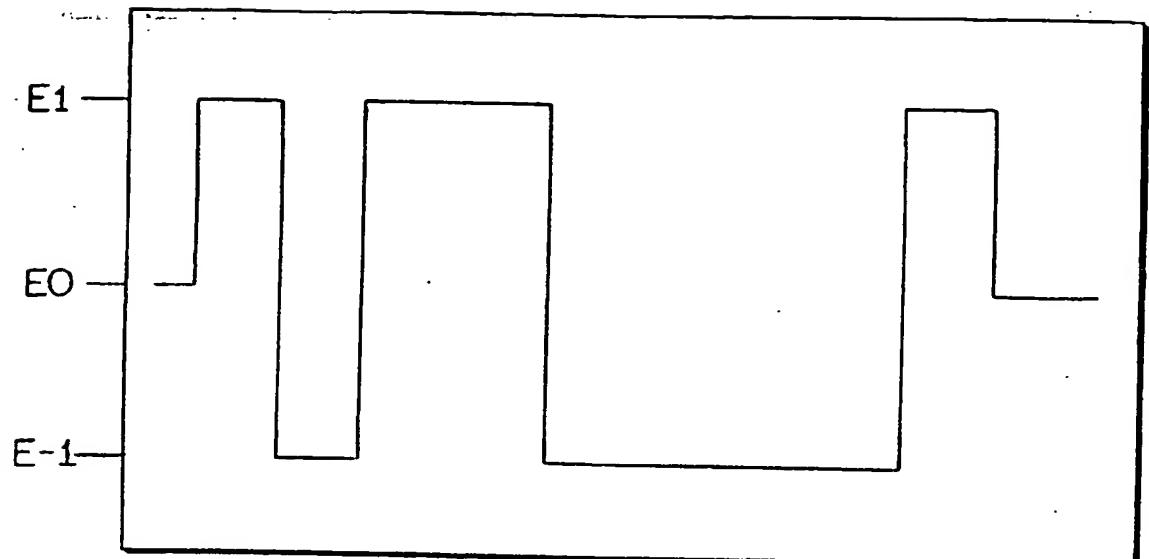


FIG. 6

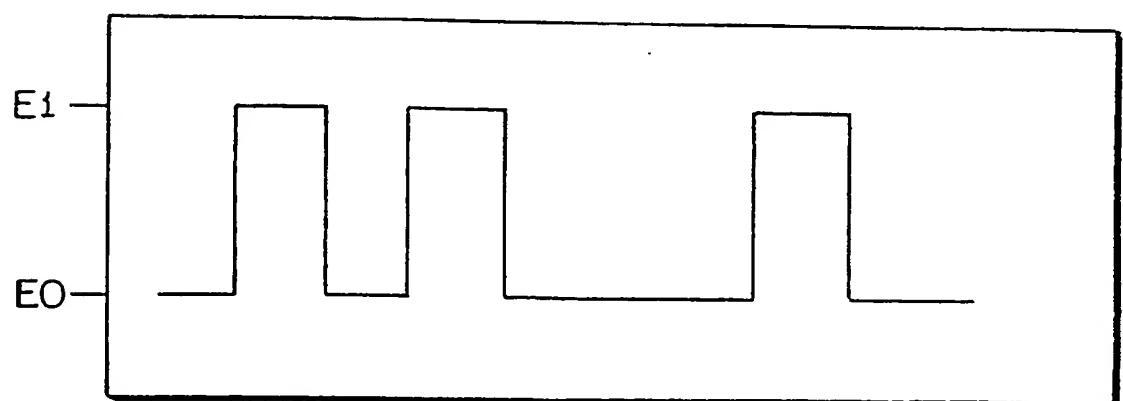


FIG. 7

3/4

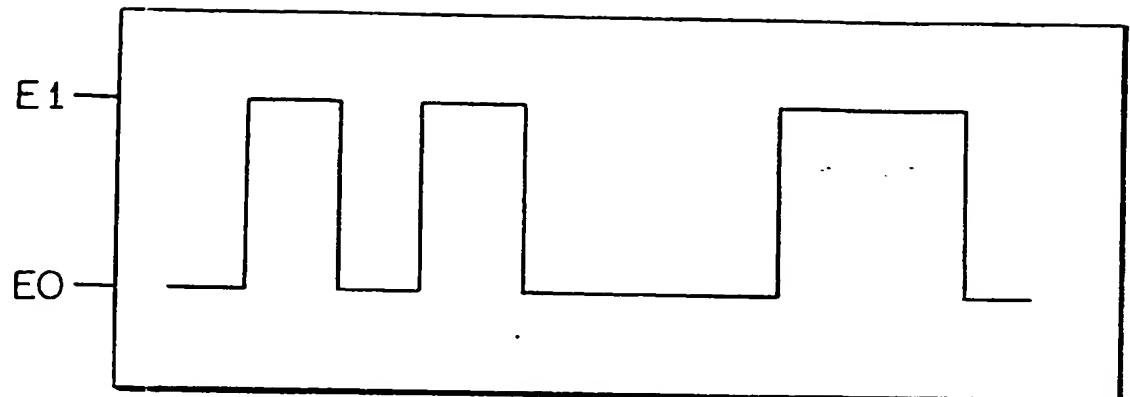


FIG. 8

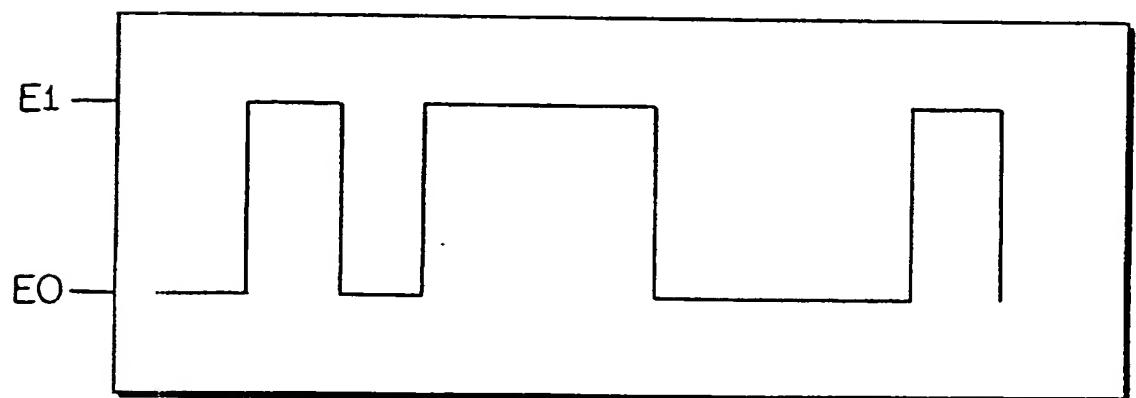


FIG. 9

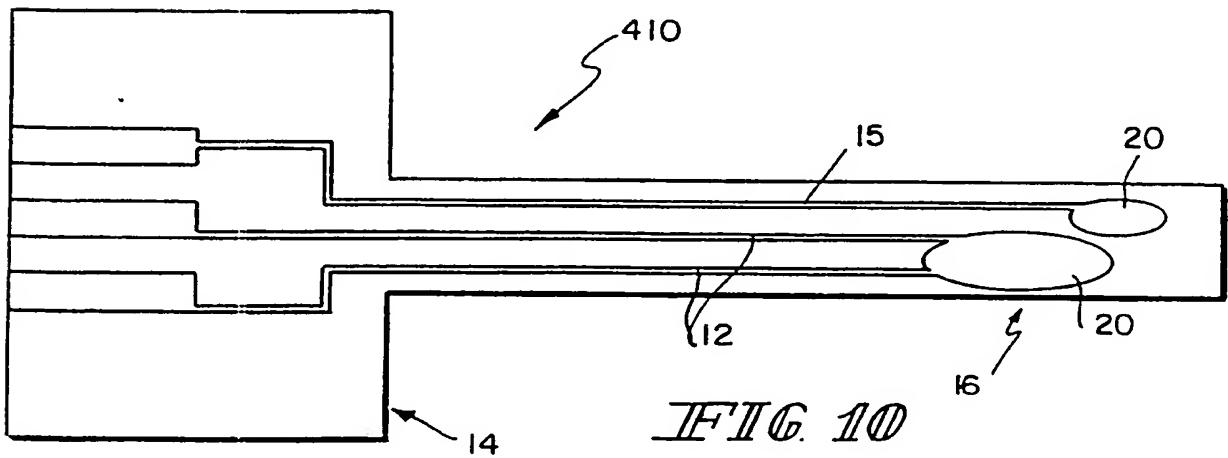


FIG. 10

Fig. 11

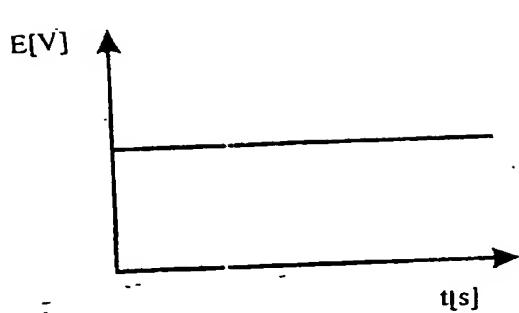


Fig. 12

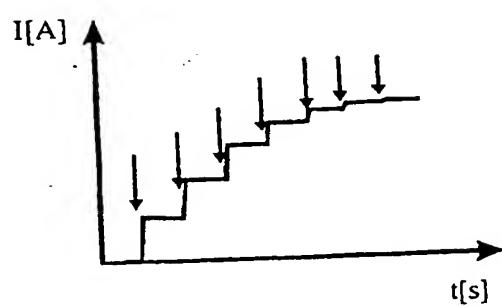
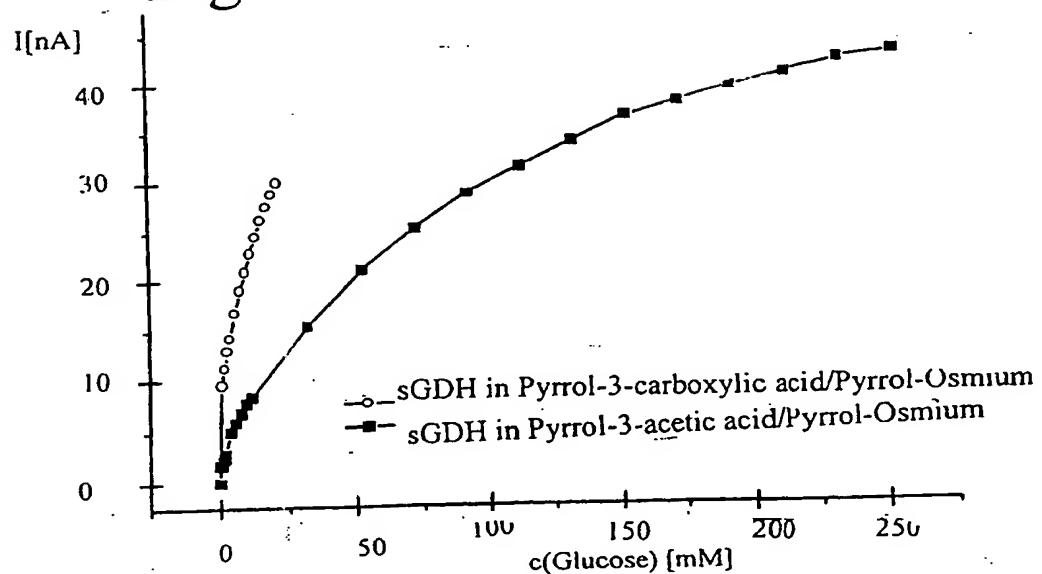


Fig. 13



INTERNATIONAL SEARCH REPORT

Int'l. Appl. No.

PCT/US 00/25631

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 35225 A (TOMASCO MICHAEL F ; HELLER ADAM (US); HELLER E & CO (US); SAY JAMES) 13 August 1998 (1998-08-13)	1-6, 10-17, 21
Y	abstract page 12, line 30 -page 13, line 8 page 37, line 3 - line 8 page 40 -page 41; example 3 page 42, line 18 - line 21 page 46 -page 51; claims 1,8,11-26,33,36,41-45 page 56 -page 58; claims 82,89-94 figure 10	23-27
A	---	7-9, 18-20 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

19 February 2001

Date of mailing of the international search report

27/02/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2260 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Tuynman, A

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/25631

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 29199 A (BIO MERIEUX ;GARNIER FRANCIS (FR)) 2 November 1995 (1995-11-02) figures 2A,3 page 11; example 1 page 13, line 26 -page 14, line 2 ----	23-27
A	WO 93 06237 A (ALLAGE ASSOCIATES INC) 1 April 1993 (1993-04-01) page 28 -page 29; claims 1-5 page 12, line 30 -page 14, line 28 ----	1,7-10, 16-21
A	WO 98 58250 A (ELAN CORP PLC) 23 December 1998 (1998-12-23) page 14, line 14 -page 15, line 26 page 24, line 1 -page 25, line 6 ----	1,2, 7-10, 16-21
A	US 5 735 273 A (KURNIK RONALD T ET AL) 7 April 1998 (1998-04-07) column 16, line 10 -column 17, line 27 ----	7-10, 16-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. onal Application No

PCT/US 00/25631

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9835225	A 13-08-1998	AU 6157898	A 26-08-1998	EP 0958495	A 24-11-1999
		JP 2000509507	T 25-07-2000	US 6143164	A 07-11-2000
		US 6120676	A 19-09-2000		
WO 9529199	A 02-11-1995	FR 2720832	A 08-12-1995	CA 2165978	A 02-11-1995
		EP 0705291	A 10-04-1996	JP 9504334	T 28-04-1997
		US 6096825	A 01-08-2000		
WO 9306237	A 01-04-1993	US 5312762	A 17-05-1994		
WO 9858250	A 23-12-1998	IE 970443	A 16-12-1998	AU 8031898	A 04-01-1999
		EP 0990151	A 05-04-2000	TW 380046	B 21-01-2000
		ZA 9805189	A 08-01-1999		
US 5735273	A 07-04-1998	AU 703849	B 01-04-1999	AU 6497396	A 01-04-1997
		CA 2229509	A 20-03-1997	EP 0876501	A 11-11-1998
		JP 11512316	T 26-10-1999	WO 9710356	A 20-03-1997
		US 6141573	A 31-10-2000	US 5827183	A 27-10-1998